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The different pathways of spore germination and inactivation of *Bacillus subtilis* under high pressure and elevated temperatures

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Abstract

High pressure combined with elevated temperatures could produce commercially sterile and shelf stable low acid foods. Depending on the applied temperature and pressure level, bacterial endospores pass through different physiological pathways, which can induce germination or a subsequent inactivation. To improve the understanding of spore inactivation *Bacillus subtilis* spores and isogenic strains, which lack the gens for encoding the germinant receptors, were pressure treated. Therefore, the strains *FB114* and *FB115* and its wild type strain (*PS832*) were processed from 1 s up to 24 h in a pressure-temperature range of 200 – 1000 MPa and 30 - 80°C under isothermal and isobaric conditions during dwell time in pressure stable ACES buffer solution. Afterwards, aliquots of each sample were mild heat treated and the released amount of DPA was quantified by HPLC, to estimate the amount of germinated spores. Further, two model approaches were tested to derive a global model from the kinetic data. It was confirmed for both FB-strains, that spore germination above 500 MPa is possible without germinant receptors and even at 300 MPa germination of these spores was detected. Furthermore, no spore inactivation occurred for pressures below 700 MPa and temperatures up to 50 °C. Whereas the behavior of PS832 was contrary, here an inactivation of more than 3 $\log_{10}$-cycles was detected at 300 MPa after 30 min dwell time at 40°C and no inactivation was observed at 550 MPa, 37°C, 120 min. Possibly, spores with nutrient receptors could proceed through stage II of germination at low pressure levels, whereas this germination step is retarded at higher pressures. Moreover, above 300 MPa both germination mechanisms interfere with each other, whereas above 500 MPa the opening of the Ca^{2+}-DPA-channels is dominant. For all strains a strong acceleration of germination with increasing pressure and temperature was detected, whereas above a certain threshold pressure ($p > 600$ MPa) the temperature played the dominant role for spore inactivation.

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Keywords: High pressure thermal sterilization; bacterial spores; germiantion- and ianctivation mechanisms; modeling.

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1. Introduction

Isostatic high pressure (HP) in the food industry is primarily used for the pasteurization of foods at chilled and ambient temperatures. It has been applied on a broad range of products. However, to achieve a sterilization of "low acid foods" HP must be combined with an elevated initial temperature (> 80 °C) to sufficiently inactivate bacterial spores. This High Pressure Thermal Sterilization (HPST) treatment can result in sterile food products with reduced thermal load, higher nutritional quality and increased functionalities, rendering HP promising improvement from traditional heat sterilization [1, 2].

At ambient temperatures, ungerminated bacterial spores are highly resistant to pressure. They need at least 800 to 1400 MPa [3] to be inactivated, which is far more than what is commercially feasible. However, in dependence of the applied temperature and pressure level, bacterial endospores pass through different physiological pathways, which could induce spore germination or a subsequent inactivation during the treatment. For various *Bacillus* ssp. spores, inactivation has proven more efficient at ambient temperature and moderate pressure (200 MPa < p > 500 MPa) than at higher pressures (p > 500 MPa) [4, 5]. These authors have argued that pressure-induced non-nutrient germination may explain this increase in efficiency as it activates the germination receptors of the spores, making them more vulnerable to pressure and heat [6]. This HPST treatment is unlikely for the commercial treatment of low acid foods, however, as the extension of spore germination is strongly time- and temperature-dependent, and more importantly, because full germination of the whole entire spore population is not possible [4, 5]. An additional increase in pressure (p > 500 MPa) activates spore germination by instigating changes in the spore´s inner membrane. This process triggers the spores to release both the large depots of dipicolinic acid (DPA) and the associated divalent cations (predominantly Ca^{2+}) from their core via an opening in the spores' Ca^{2+}-DPA channels [6]. Hence, spores that lack their nutrient receptors could also germinate at these very HP [7]; however, the complete inactivation mechanism of bacterial spores is still not fully understood. Inactivation studies of *Geobacillus stearothermophilus* spores yielded the highest heat sensitivity at approximately 700 MPa; HP stabilized the spores, inhibiting them from heat-induced inactivation under pressure [2]. Similar results were found for *B. amyloliquefaciens* [8] and other strains of *G. stearothermophilus* spores [9].

Consequently, inactivation kinetics and the mechanisms of bacterial spores have to be explored to guarantee, that the sterilization process is safe [2, 8, 10].

2. Materials & Methods

2.1. Spore preparation and description of the used spore strains

The spore strains used for this study were all isogenic derivates of the *Bacillus subtilis* strain 168. In the strains FB 114 and FB115, all genes responsible for the encoding of the germinant receptors were inactive. Hence, the used spore strains can not germinate in the presence of nutrients, but could be germinated at pressures above 500 MPa [7]. Further, both strains lack one of the two cortex lytic enzymes (CLE) *sleB* and *CwlJ*, namely *sleB* in the strain FB 114 and *CwlJ* in FB 115, respectively. The strain PS832 is a wild-type strain of *Bacillus subtilis* and was used in this study to evaluate the impact of the pressure treatment, if nutrient receptors are present.

The sporulation was induced at 37 °C on solid 2x SG medium agar plates without antibiotics. The harvested spores were cleaned by repeated centrifugation (5000 g) and washing with cold distilled water. The resulting spore suspensions contained ≥ 95 % phase bright spores and nearly no spore agglomerates checked by flow particle image analysis (FPIA 3000, Malvern Instruments Ltd., Worcestershire, UK).

2.2. Pressure treatment and determination of viable spores

For pressure treatment all three spore strains were diluted in pressure stable ACES buffer solution (pH 7, 0.05 M) with an initial cell count of approximately 1.0 x 10^8 CFU mL^{-1} and split into small aliquots of 1.6 mL. These samples were then filled into plastic tubes (CryoTube Vials, Nunc Brand Products, Rosklide, Denmark) or split again into 300 µL and pipetted into shrinking tubes (Schrumpfschlauch 3/1, DSG-Canusa, Meckenheim, Germany) with an inner diameter of 3 mm and an outer diameter of 3.6 mm. Finally, the tubes were hermitically sealed with a soldering iron. All samples were stored on ice prior to, and following pressure treatment.

An HP sterilization unit (0101-7000-S, Sitec Sieber Engineering AG, Zurich, Switzerland), with a maximum vessel volume of 100 mL and a compression rate of 4 MPa s^{-1}, was used for pressure treatment of the plastic tubes (1.6 mL) in the pressure temperature range of 200 MPa – 700 MPa and 30°C-80°C. For pressure treatments between 600 MPa and 1 GPa (T = 60°C - 80°C), a HP unit (U111 Unipress, Warsaw, Poland), with a compression rate of 25 MPa s^{-1} and a vessel volume of 3.7 mL, was used. The HP vessels were preheated to the designated treatment temperature with a thermostatic bath (Huber GmbH, Offenburg, Germany). The samples of all three spore strains were treated simultaneously under isothermal and isobaric conditions during dwell time. Another plastic tube was used as a dummy container - equipped with a thermocouple in the geometrical center of the tube - to determine the heat of compression during pressure build-up. The cooled samples and the dummy sample (~ 2°C) were preheated to the empirically determined starting temperature in the HP vessel. After pressure treatment, aliquots of each sample were treated for 20 min at 80°C to determine the number of germinated, and therefore heat sensitive spores [5].

Following thermal treatment, the samples were immediately transferred into an ice bath. Afterwards, they were serially diluted with ¼ Ringer solution (TP887925 712, Merck KGaA, Darmstadt, Germany) in 96-well microtiter plates (Carl Roth GmbH, Karlsruhe, Germany), and two 50 µL samples of every dilution were spread-plated in petri dishes on nutrient agar (CM 003, Oxoid Ltd., Hampshire, England). The dishes were incubated at 37°C for 2 days and the colonies were counted. Due to the strains' inability to germinate on nutrient agar plates without pressure treatment, a counting chamber (Thoma counting chamber, Paul Marienfeld GmbH & Co. KG, Lauda- Königshofen, Germany) was used to determine the total cell count of strains FB114 and FB115.

2.3. Global model calculation and evaluation

In order to generate a pressure-temperature isorate diagram, different modeling approaches were tested for its applicability. Based on the germination and inactivation data for isobaric and isothermal conditions (t_0 = 1 s dwell time), an empirical model, which is analogue to the n^{th}-order approach for chemical degradation reactions, was used:

$$\log\frac{N}{N_0} = \frac{1}{1-n}\log\left(1 + k \cdot N_0^{(n-1)} \cdot t \cdot (n-1)\right) \qquad (1)$$

with N and N_0 = spore counts (CFU mL^{-1}) at time t and t_0, respectively, t = time, k = inactivation rate constant and n = reaction order. This equation proved to be suitable for the description of spore inactivation under isobaric and isothermal inactivation [11]. Further, an alternative model for nonlinear spore survival curves was tested for the development of a global model. This model is based on the Weibull model, but it allows additionally a modeling of shoulder formations, which often occurs in thermal or combined pressure and thermal inactivation kinetics of bacterial spores.

$$\log S(t) = b_1 \cdot t^{n_1} - b_2 \cdot t^{n_2} \qquad (2)$$

The first part of the right-hand side refers to the activation part of the survival curve, and the second part to the inactivation part of the survival curve, with log $S(t)=N/N_1$ (with N_1 as the initial inactivation after 1 s dwell time), b_1 and b_2 as scale parameters and n_1 and n_2 as the so called shape parameters[12]. Obviously, if there is no shoulder, which implies that $b_1 = 0$, the model reduces to the Weibullian model.

The non-linear regression for each individual kinetic curve was performed with TableCurve 2D (SPSS Inc., Chicago, IL, USA), the isokineticity lines were calculated with MathCAD 15 (Mathsoft Engineering & Education, Inc., USA) and plotted with OriginPro (Version 8.0724, B724; OriginLab Corporation, Northampton, MA, USA)

3. Results & Discussion

It was confirmed for both FB-strains that spore germination above 500 MPa is possible without nutrient germinant receptors [7], whereas a strong acceleration of the germination rate was observed with increasing process temperature.

Interestingly, even at 200 MPa and 30°C a germination of both FB-strains was detected by an increase in viable spore count on Nutrient-Agar plats of around 1 $\log_{10}$ cycle after 20 min dwell time in comparison to the unpressurized sample. However, to induce a germination of nearly 100% in the pressurized FB-spore suspensions 300 MPa and 40°C were needed. Hence, this is the first report of pressure induced germination without nutrient receptors below 500 MPa. The mechanism for this non-nutrient germination is extensively discussed in literature and most of the authors assumed a direct opening of the DPA-Ca^{2+}-Channels at pressures above 500 MPa [2, 5-7, 13]. Due to the long pressure dwell times applied in this study in comparison to pressure dwells of less than 20 min in other studies, we assume as well that this germination is probably due to a direct opening of DPA-Ca^{2+} channels and that consequently the critical pressure level for this germination mechanism at ambient temperature has to be lowered to 300 MPa.

Furthermore, no spore inactivation occurred for pressures below 700 MPa and treatment temperatures up to 50°C, which indicates that these pressure temperature conditions are sufficient enough to induce a germination in spores without germination receptors, but that they are not suitable to inactive them.

Contrary, the behavior of the wild type spore strain (PS832), where an inactivation of more than 3 $\log_{10}$ was detected at 300 MPa after 30 min dwell time at 40°C and no inactivation was observed at 550 MPa, 37°C, 120 min. This could be due to, that spores with nutrient receptors could proceed through stage II of germination at low pressure levels [6], whereas this germination step is retarded at higher pressures.

For all spore strains a strong acceleration of the spore germination with increasing pressure and temperature was detected, whereas above a certain threshold pressure ($p \geq 600$ MPa) the treatment temperature played the prominent role for spore inactivation (Figure 2 A). For instance at 60°C a constant 3 $\log_{10}$ inactivation was achieved at 600 – 900 MPa within 15 min, whereas an temperature increase from 60°C to 80°C at 600 MPa reduced the dwell time to 2 min (Figure 2 A). Further, the amount of germinated spores (detect by the amount of thermal sensitive spores) was always higher as the amount of inactivated spores (Figure 2 B), which confirms that spore inactivation under pressure is a minimum two-step inactivation mechanism, including first a germination and afterwards an inactivation under pressure.

To connect all individual inactivation kinetics into a global model, a part of the generated data (600 MPa – 1 GPa and 60°C-80°C), which were all obtained in the U111 HP unit for the wild-type spore strain PS832 were used for a first ”test- modeling”. Two different models, an n^{th}-order model and a double Weibullian model, were tested for its applicability to calculate isorates for a fixed spore inactivation in a pressure-temperature diagram.

For the double Weibullian model (Eqn. 2) first of all, an individual non-linear regression fit of every inactivation kinetic was done to determine the scale and shape parameters. The individual fit of each kinetic was quite good, but no dependence of the parameters b_1, b_2, n_1 and n_2 on pressure and temperature

could be identified. However, a non systematic variation of the shape and scale parameters is not unusual and quite often reported for thermal inactivation kinetics [12]. Hence, it was assumed that n_1 and n_2 are constant with varying pressure and temperature and the average value for n_1 = 1.12 and n_2 =1.14 was used for a second non-linear regression (Table 1).

Table 1. Individual values for the scale parameters b_1 and b_2 for fixed shape parameters for n_1=1.12 and n_2= 1.14

Pressure [MPa]	Temperature [°C]	b_1	b_2	R^2	std
600	60	-0.00992	-0.00750	0.979	0.367
	70	0.04915	0.04739	0.976	0.428
	80	-0.23518	-0.20232	0.952	0.693
700	60	0.00515	0.00548	0.979	0.347
	70	-0.16545	-0.14178	0.971	0.456
	80	0.15784	0.16095	0.975	0.536
800	60	-0.00368	-0.00215	0.984	0.294
	70	-0.01023	-0.00360	0.958	0.528
	80	-0.46202	-0.40262	0.881	1.256
900	60	0.04729	0.04248	0.996	0.123
	70	-0.11702	-0.09811	0.925	0.754
	80	0.53429	0.51974	0.996	0.156
1000	70	0.10953	0.10521	0.996	0.156
	80	-0.05553	-0.01813	0.956	0.533

This approximation of fixed values for the shape parameters even increase the goodness of fit, but again no systematic variation of b_1 and b_2 could be identified (Figure 1). Consequently, this modeling approach proofed to be suitable to fit the individual inactivation kinetics quite well, but failed to derive a global model for the inactivation over a broad pressure temperature range.

Furthermore, the strong correlation of the parameter b_1 and b_2 as well as n_1 and n_2, respectively, is pointing towards that the simple Weibullian model will achieve a comparable goodness of fit.

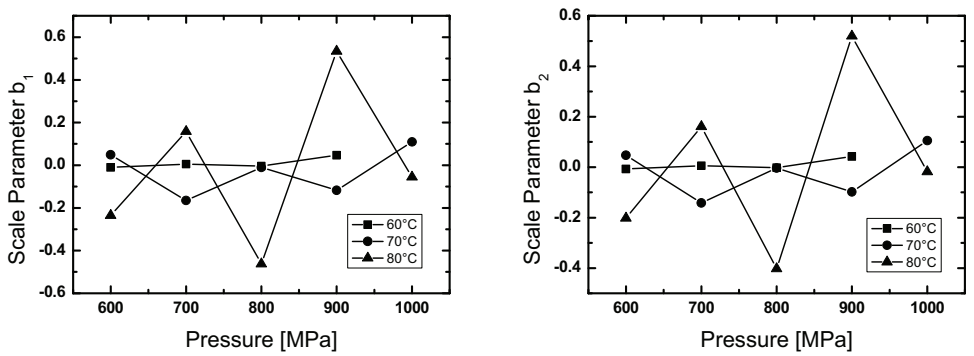

Fig. 1. Pressure dependence of the scale parameters b_1 and b_2 for fixed shape parameters for n_1=1.12 and n_2= 1.14

The second model tested, was the n^{th}-order approach. To identify the reaction order, all individual kinetics were fitted over a range of reaction orders (n = 1.0 – 1.7). The minimal cumulative standard error (Σ SD) was used to identified the optimal reaction order of n=1.05 for the pressurized samples or n = 1.2 for the additionally thermal treated spores. After the identification of the reaction order (n), the rate constants (k) were obtained regressively. To get a functional relationship of the rate constant with pressure and temperature dependence [k(p,T)], empirical equations have often been suggested [8, 14]. Hence, a taylor series expansion up to 2nd order terms,

$$\ln k(p,T) = a+bp+cT+dp^2+eT^2+fpT+gp^2T+hpT^2 \quad (3)$$

was used to calculate the rate constant k(p,T). To solve the functional relationship between pressure and temperature, k in equation 1 was replaced with k(p,T) (Eqn. 3) and the reduction rate (N/N_0) and time (t) were set as constants and isorate lines for a fixed inactivation could be calculated (Figure 2).

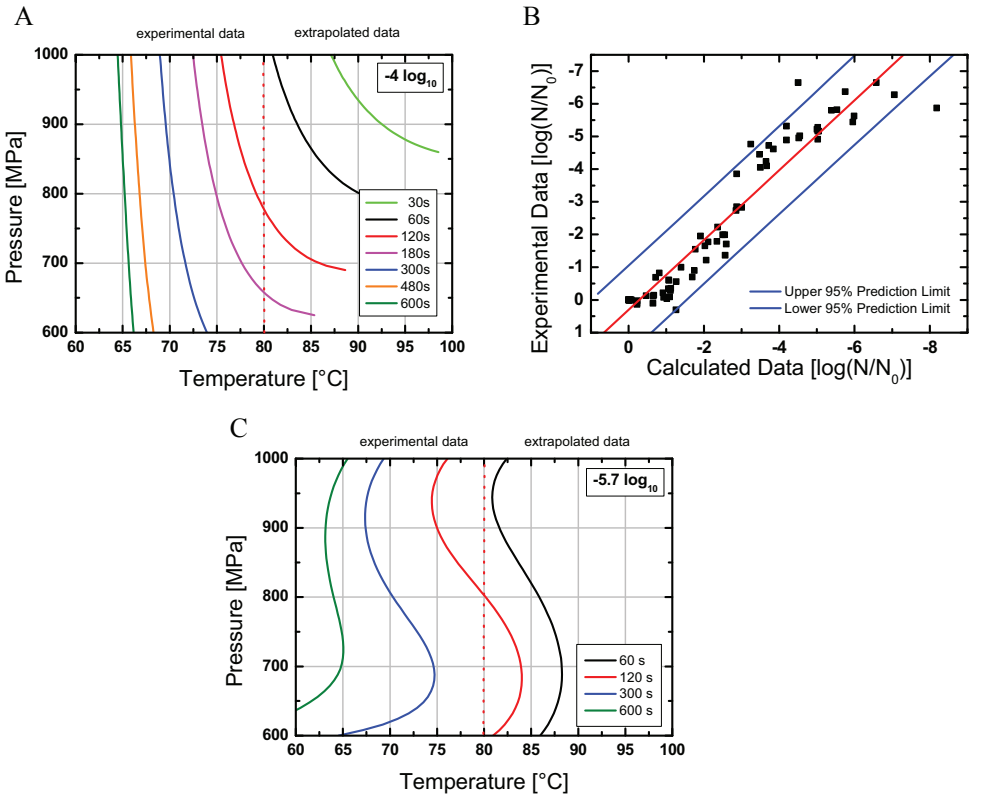

Fig. 2. A) Isorate lines (n=1.05) for a 4 $\log_{10}$ inactivation of *Bacillus subtilis* (PS832) spores in pressure stable 0.05 M ACES buffer solution (pH 7) after 30 s-600 s at isobaric/ isothermal conditions with N (1 s) as initial population N_0. B) Comparison between calculated data and experimental $\log_{10}$ reduction of *Bacillus subtilis* (PS832) with n = 1.05. C) Isorate lines (n=1.2) for a 5.7 $\log_{10}$ inactivation (detection limit) of *Bacillus subtilis* (PS832) spores in pressure stable 0.05 M ACES buffer solution (pH 7) after 30 s-600 s at isobaric/ isothermal conditions plus an additional heat treatment (20 min at 80°C) with N (1 s) as initial population N_0

The overall fit of the n^{th}-order model for the only pressure treated spores (Figure 2A) was acceptable, but Figure 2B displays that the residuals are not normal distributed, which indicates a heteroscedastic

error. This error distribution is due to, that the n^{th}-order model could adequately describe inactivation curve with a pronounced tailing, but is not applicable for kinetics with shoulder and tailing. Therefore, this model (n = 1.05) is predicts an overestimated inactivation for low inactivation levels (< -2.5 $\log_{10}$) and an underestimation for higher inactivation levels. Though, for the post-thermal treated samples (Figure 2C) the goodness of fit was much better and the residual plot was randomly distributed (data not shown), due to that no shoulder formation occurred in these kinetics.

However, both isorate-diagrams display the germination and inactivation behavior of *Bacillus subtilis* (PS832) with an acceptable accuracy and confirm that above 600 MPa the treatment temperature played the prominent role for spore inactivation. Further, a strong decrease in the germination rate at around 700 MPa is obvious in Figure 2C, which is remarkably equal to the inactivation behavior of *Geobacillus stearothermophilus* under HP and ultra high temperature (T > 90°C) [2]. A possible explanation for a retarded germination at around 700 MPa is given by Reineke et al. [15], who assumed that up to 600 MPa a germination triggered by nutrient receptors interferes with a germination induced by a direct opening of the Ca^{2+}-DPA channels. With increasing treatment pressure and temperature the germination rate induced by the opening of the Ca^{2+}-DPA channels increases, whereas the impact of the germination receptors on the overall germination rate above 700 MPa could be neglected.

To derive a global model for the whole dataset, including a link between the amount of germinated spores and the subsequent inactivation step and possibly also the different germination inducing mechanism, the tested model approaches failed. Hence, other models like for instance multi-response kinetic modeling have to be tested.

4. Conclusions

The continuous increase of HP research over the last decades has already generated an impressive number of commercially available pressure pasteurized products. Furthermore, due to extensive research a US-FDA-certification of a pressure assisted thermal sterilization process is available since February 2009. However, industrial application of this promising sterilization technology is still awaiting realization. An improved understanding of spore inactivation mechanisms and the possibility to calculate the desired inactivation levels will help to introduce this technology in pilot and industrial scale.

Hence, based on these findings for the germination and inactivation of *Bacillus subtilis* spores, it could be assumed, that for the industrial scale HPST process it is only necessary to reach this threshold pressure of 600 MPa and increase further the process temperature to reduce the processing time. However, this threshold pressure is presumably spore strain specific and has to be determined individually for the target spore strain in the treated food matrix.

References

[1] Heinz, V. and D. Knorr, *Effects of High Pressure on Spores*, in *Ultra High Pressure Treatments of Foods*, M. Hendrickx and D. Knorr, Editors. 2002, Kluwer Academic / Plenum Publishers: New York. p. 77-114.

[2] Mathys, A., et al., High pressure thermal sterilization - development and application of temperature controlled spore inactivation studies. *High Pressure Research*, 2009. **29**(1): p. 3-7.

[3] Reineke, K., et al., Temperature control for high pressure processes up to 1400 MPa. *Journal of Physics: Conference Series,*, 2008. **121**: p. 142012-142016.

[4] Heinz, V. and D. Knorr, *High pressure germination and inactivation kinetics of bacterial spores*, in *High pressure food science, bioscience and chemistry*, N.S. Isaacs, Editor. 1998, The Royal Society of Chemistry: Cambridge. p. 435-441.

[5]Wuytack, E.Y., S. Boven, and C.W. Michiels, Comparative study of pressure-induced germination of *Bacillus subtilis* spores at low and high pressures. *Applied and Environmental Microbiology*, 1998. **64**(9): p. 3220-3224.

[6] Setlow, P., Spore germination. *Current Opinion in Microbiology*, 2003. **6**: p. 550–556.

[7] Paidhungat, M., et al., Mechanisms of induction of germination of *Bacillus subtilis* spores by high pressure. *Applied and Environmental Microbiology*, 2002. **68**(6): p. 3172-3175.

[8] Margosch, D., et al., High-pressure-mediated survival of *Clostridium botulinum* and *Bacillus amyloliquefaciens* endospores at high temperature. *Appl. Environ. Microb.*, 2006. **72(5)**: p. 3476-3481.

[9] Ardia, A., *Process Considerations on the Application of High Pressure Treatment at Elevated Temperature Levels for Food Preservation*, in *Department of Food Biotechnology and Food Process Engineering*. 2004a, Berlin University of Technology: Berlin. p. 94.

[10] Heinz, V. and D. Knorr, High pressure inactivation kinetics of *Bacillus subtilis* cells by a three-state-model considering distribution resistance mechanisms. *Food Biotechnology*, 1996. **10**(2): p. 149-161.

[11] Knorr, D., et al., *High-Pressure-Induced Effects on Bacterial Spores, Vegetative Microorganisms, and Enzymes*, in *Food Engineering Interfaces*, J.M. Aguilera, et al., Editors. 2011, sPRINGE: New York. p. 325-340.

[12] Van Boekel, M.A.J.S., *Kinetic Modeling of Reactions in Foods*. 2009, London: CRC Press.

[13] Black, E.P., et al., Analysis of factors influencing the rate of germination of spores of *Bacillus subtilis* by very high pressure. *Journal of Applied Microbiology*, 2006. **102**(1): p. 65-76.

[14] Ardia, A., et al., Kinetic studies on combined high-pressure and temperature inactivation of *Alicyclobacillus acidoterrestris* spores in orange juice. *Applied Biotechnology, Food Science and Policy*, 2004. **1**(3): p. 169-173.

[15]Reineke, K., A. Mathys, and D. Knorr, The impact of high pressure and temperature on bacterial spores- Inactivation mechanisms of *Bacillus subtilis* above 500 MPa. *Journal of Food Science*, 2011. **76**(3): p.189-197.

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